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Induction of Apoptotic Program in Cell-Free Extracts: Requirement for dATP and Cytochrome c

Xuesong Liu,* Caryn Naekyung Kim,* Jie Yang,* Ronald Jemmerson,† and Xiaodong Wang*
*Department of Biochemistry
Emory University School of Medicine
Atlanta, Georgia 30322
†Department of Microbiology
University of Minnesota Medical School
Minneapolis, Minnesota 55455

Summary

A cell-free system based on cytosols of normally growing cells is established that reproduces aspects of the apoptotic program in vitro. The apoptotic program is initiated by addition of dATP. Fractionation of cytosol yielded a 15 kDa protein that is required for in vitro apoptosis. The absorption spectrum and protein sequence revealed that this protein is cytochrome c. Elimination of cytochrome c from cytosol by immunodepletion, or inclusion of sucrose to stabilize mitochondria during cytosol preparation, diminished the apoptotic activity. Adding back cytochrome c to the cytochrome c–depleted extracts restored their apoptotic activity. Cells undergoing apoptosis in vivo showed increased release of cytochrome c to their cytosol, suggesting that mitochondria may function in apoptosis by releasing cytochrome c.

Introduction

Apoptosis is a distinct form of cell death controlled by an internally encoded suicide program (reviewed by Steller, 1995; White, 1996). The morphologic changes associated with apoptosis include condensation of nucleoplasm and cytoplasm, blebbing of cytoplasmic membranes, and fragmentation of the cell into apoptotic bodies that are rapidly phagocytosed by neighboring cells (Kerr, 1971; Wyllie et al., 1980). The biochemical markers of apoptosis include DNA fragmentation into nucleosomal fragments (Wyllie, 1980), activation of the interleukin-1β-converting enzyme (ICE) family of proteases (Schlegel et al., 1996; Duan et al., 1996; Wang et al., 1996), and cleavage of various substrates of the ICE family of proteases, including poly(ADP-ribose) polymerase (PARP) (Tewari et al., 1995; Nicholson et al., 1995), sterol-regulatory element–binding proteins (SREBPs) (Wang et al., 1995, 1996), nuclear lamin (Lazebnik et al., 1995), and the U1 associated 70-kDa protein (Casciola-Rosen et al., 1994).

The cell suicide program is best illustrated by genetic studies in the nematode Caenorhabditis elegans (Hengartner and Horvitz, 1994b). Two genes involved in the control of programmed cell death in C. elegans have been well characterized. One gene, *ced-9*, encodes a protein that prevents cells from undergoing apoptosis (Hengartner et al., 1992), while another gene, *ced-3*, encodes a protease whose activity is required to initiate apoptosis (Yuan and Horvitz, 1990).

The *bcl-2* family of genes are the mammalian counterparts of *ced-9* (Hengartner and Horvitz, 1994a). Overexpression of *bcl-2* prevents mammalian cells from undergoing apoptosis in response to a variety of stimuli (reviewed by Reed, 1994). The bcl-2 protein is located primarily on the outer membranes of mitochondria (Monaghan et al., 1992; Krajewski et al., 1993; de Jong et al., 1994), suggesting the involvement of mitochondria in apoptosis. However, the biochemical mechanism of bcl-2 function is still obscure.

The ced-3 protein is a cysteine protease related to the ICE family of proteases in mammalian cells (Yuan et al., 1993). The closest mammalian homolog of ced-3 is CPP32 (Fernandes-Alnemri et al., 1994), which cleaves PARP and SREBPs in cells undergoing apoptosis (Tewari et al., 1995; Nicholson et al., 1995; Wang et al., 1996). CPP32 is closely related to ced-3 in terms of sequence identity and substrate specificity (Xue and Horvitz, 1995). Like ced-3 in C. elegans, CPP32 normally exists in the cytosolic fraction as an inactive precursor that becomes activated proteolytically in cells undergoing apoptosis (Schlegel et al., 1996; Wang et al., 1996). More direct evidence to support the concept that the activity of CPP32 is required for apoptosis emerged from an experiment in which a tetrapeptide aldehyde inhibitor that specifically inhibits CPP32 activity was shown to block the ability of cytosol from apoptotic cells to induce apoptosis-like changes in normal nuclei in vitro (Nicholson et al., 1995).

The ability of activated CPP32 to trigger apoptosis implies that cells must have a highly regulated mechanism to control this activation to prevent unwanted cell death. CPP32 is activated by multiple proteolytic cleavages of its 32-kDa precursor form to generate the 17/11-kDa or 20/11-kDa active form (Nicholson et al., 1995; Wang et al., 1995). Since CPP32 is activated by cleavage at aspartic acid residues, a hallmark of ICE-like proteases (Thornberry et al., 1992), a cascade of ICE-like proteolytic cleavages leading to apoptosis has been proposed (Tewari et al., 1995; Wang et al., 1996). Activated CPP32 from HeLa cell extracts was able to cleave the CPP32 precursor (Wang et al., 1996), indicating that CPP32 can be activated through autocatalysis. A similar mechanism is probably responsible for yielding the active enzyme when the CPP32 precursor is expressed in large quantity in bacteria (Xue and Horvitz, 1995). Recently, another ICE family protease has been identified that may be responsible for cleaving the CPP32 precursor into the 20/11-kDa active form. This enzyme has been purified from hamster liver extracts and identified as the hamster homolog of Mch2α (Liu et al., 1996; Fernandes-Alnemri et al., 1995a). Autocatalysis and the protease cascade may provide the signal amplification necessary for rapid and irreversible apoptosis, but the intracellular factors that trigger this amplification have yet to be identified.

There have been several previous reports of cell-free apoptosis systems that induce apoptotic changes in the

added nuclei (Lazebnik et al., 1993; Newmeyer et al., 1994; Eeari et al., 1995; Martin et al., 1995). These systems require cytosol from cells that were already undergoing apoptosis in vivo, so they cannot be used to detect triggering factors.

In the current study, we establish a cell-free system that duplicates the features of the apoptotic program, including activation of CPP32 and DNA fragmentation. Apoptosis in this system is initiated by the addition of deoxyadenosine-5-triphosphate (dATP). This system allowed us to fractionate and purify the biochemical components that trigger the activation of the apoptotic proteases and DNA fragmentation. One of the protein factors has been purified to homogeneity and identified as cytochrome c.

Results

dATP-Dependent Activation of CPP32 and DNA Fragmentation

Activation of CPP32 and DNA fragmentation are two of the well characterized biochemical markers of apoptosis. In an effort to establish an in vitro system that duplicates apoptosis, we prepared 100,000 g cytosolic supernatant (S-100) from suspension cultures of HeLa cells. Since the activation of CPP32 is the result of cleavage of its 32-kDa precursor into the 20-kDa NH_2-terminal fragment and 11-kDa COOH-terminal fragment (Nicholson et al., 1995), the activation of CPP32 in the HeLa cell S-100 was monitored by Western blot analysis using a monoclonal antibody against the 20-kDa fragment of CPP32 (Figure 1A). The enzymatic activity of CPP32 was assayed by measuring the cleavage of two ^{35}S-labeled substrates, PARP (Figure 1B) and SREBP-2 (Figure 1C). DNA fragmentation was assayed by incubating the HeLa cell S-100 with nuclei isolated from hamster liver followed by genomic DNA extraction and analysis by agarose gel electrophoresis. We found that dATP markedly accelerated the activation of CPP32 in the HeLa cell S-100. As shown in Figure 1, no activation of CPP32 was observed after the HeLa cell S-100 was incubated alone or in the presence of 1 mM ATP at 30°C for 1 hr (Figure 1A, lanes 1 and 2). However, in the presence of 1 mM dATP, most of the CPP32 in the HeLa cell S-100 was activated (Figure 1A, lane 3). The activated extracts readily cleaved PARP into 85-kDa and 24-kDa fragments (Figure 1B, lane 3) and SREBP-2 into 55-kDa and 70-kDa fragments (Figure 1C, lane 3). The sizes of the cleaved products of PARP and SREBP-2 were the same as observed in cells undergoing apoptosis (Kaufmann et al., 1993; Wang et al., 1996). It is likely that this cleavage was the result of the activation of CPP32, and related enzymes such as SCA2/Mch3, which are known to cleave PARP and SREBPs at these positions (Fernandes-Alnemri et al., 1995b; Pai et al., 1996). Consistent with the activation of CPP32, the HeLa cell S-100 in the presence of dATP induced DNA fragmentation when incubated with hamster liver nuclei (Figure 1D, lane 3). The fragmentation did not occur with HeLa S-100 alone or in the presence of ATP, confirming the requirement for dATP (Figure 1D, lanes 1 and 2).

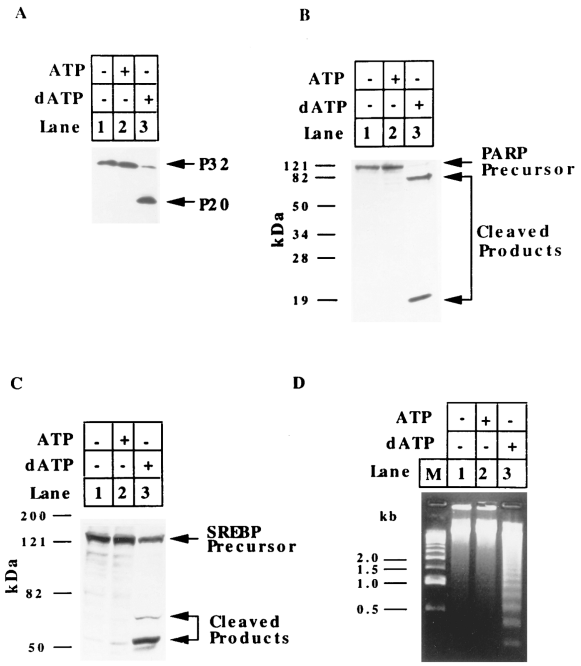

Figure 1. dATP-Dependent Activation of CPP32 and DNA Fragmentation In Vitro

Aliquot (10 μl) of HeLa cell S-100 (50 μg) was incubated alone (lane 1), or in the presence of 1 mM ATP (lane 2), or 1 mM dATP (lane 3) at 30°C for 1 hr in a final volume of 20 μl of buffer A.

(A) Samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-CPP32 antibody. The antigen/antibody complex was visualized by the ECL method as described in the Experimental Procedures. The filter was exposed to a Kodak X-OMAT AR X-ray film for 1 min.

(B) Aliquot of 10 μl of in vitro translated, ^{35}S-labeled PARP was added to each reaction. After an additional 5 min, the samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter. The filter was exposed to film for 2 hr at room temperature.

(C) Aliquot of 5 μl in vitro translated, ^{35}S-labeled SREBP-2 was added to each reaction. After incubation at 30°C for 30 min, the samples were subjected to SDS–PAGE. The gel was dried and exposed to film for 2 hr at room temperature.

(D) Aliquot (50 μl) of HeLa cell S-100 (250 μg) was incubated with 6 μl of hamster liver nuclei in the absence (lane 1) or presence (lane 2) of 1 mM ATP or dATP (lane 3) for 2 hr at 37°C in a final volume of 60 μl of buffer A. DNA was isolated as described in the Experimental Procedures and subjected to 2% agarose gel electrophoresis. The DNA was visualized by ethidium bromide staining.

To test the nucleotide specificity for activation of CPP32 and DNA fragmentation, HeLa cell S-100 was incubated with in vitro translated, ^{35}S-labeled CPP32 in the presence of 1 mM of various nucleotides (Figure 2A). Cleavage occurred only in the presence of dATP or dADP (Figure 2A). CTP, dCTP, GTP, dGTP, UTP, dTTP, ADP, AMP, dAMP, adenosine, deoxyadenosine and cAMP could not replace dATP. An identical nucleotide specificity was observed in the DNA fragmentation assay (Figure 2B).

Fractionation of Proteins Required for dATP-Dependent Activation of CPP32

The HeLa S-100 was loaded onto a phosphocellulose column, and the flowthrough and bound fractions were

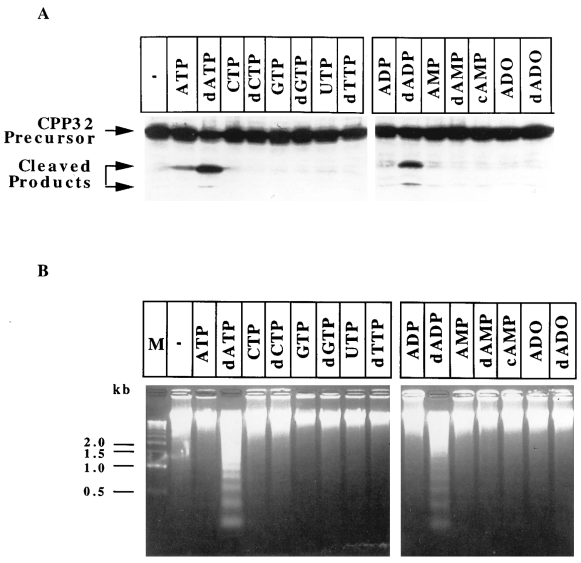

Figure 2. Nucleotide Specificity for In Vitro Activation of CPP32 and DNA Fragmentation

(A) Aliquot of 10 μl of HeLa S-100 (50 μg) was incubated with aliquot of 3 μl of in vitro translated, ^{35}S-labeled CPP32 at 30°C for 1 hr in a final volume of 20 μl in the presence of 1 mM indicated nucleotide. The samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter. The filter was exposed to film for 16 hr at room temperature.

(B) Aliquot of 50 μl of HeLa S-100 (250 μg) was incubated with aliquot of 6 μl hamster liver nuclei at 37°C for 2 hr in the presence of 1 mM indicated nucleotide. The DNA was isolated as described in the Experimental Procedures and subjected to 2% agarose gel electrophoresis. DNA was visualized by ethidium bromide staining.

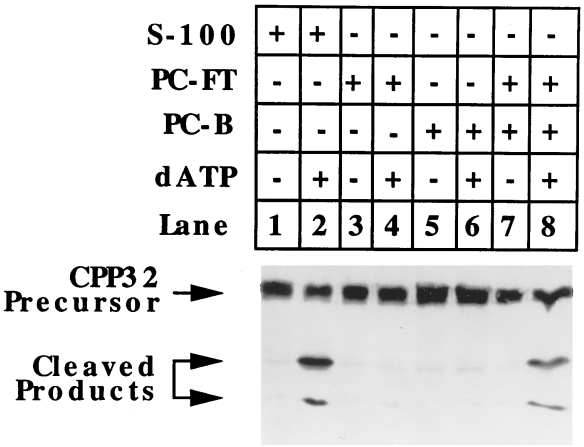

Figure 3. Fractionation and Reconstitution of dATP-Dependent Activation of CPP32 by Phosphocellulose Chromatography

HeLa cell S-100 was subjected to phosphocellulose chromatography and the column flowthrough and bound materials were collected as described in the Experimental Procedures. Aliquots (10 μl) of HeLa S-100 (50 μg) (lanes 1 and 2), phosphocellulose flowthrough fraction (PC-FT) (lanes 3 and 4), phosphocellulose bound fraction (PC-B) (lanes 5 and 6), and the mixture of phosphocellulose flowthrough and bound material (lanes 7 and 8) were incubated with aliquots of 3 μl of in vitro translated, ^{35}S-labeled CPP32 at 30°C for 1 hr in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 1 mM dATP. The samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter. The filter was exposed to film for 16 hr at room temperature.

collected. As shown in Figure 3, neither of these fractions alone supported dATP-dependent activation of CPP32 (lanes 3–6). However, when the flowthrough and bound fractions were mixed, the CPP32 activating activity was restored (lanes 7–8). The above experiment indicates that there are multiple factors contributing to the dATP-dependent activation of CPP32 that can be separated by the phosphocellulose column. The factors that flow through the phosphocellulose column are designated apoptotic protease activating factor-1 (Apaf-1), and the factor that bound to the column is designated apoptotic protease activating factor-2 (Apaf-2).

Purification of Apaf-2

The activity of Apaf-2 was assayed by recombining with Apaf-1 after purification by the following steps. First, the Apaf-2 fraction was subjected to 50% ammonium sulfate precipitation. All of the activity remained in the supernatant while most of the protein precipitated (Table 1). The supernatant was loaded onto a phenyl sepharose column, and the activity was eluted with 1 M ammonium sulfate. The eluate was passed through a gel filtration column, and active fractions were subjected to sequential Mono Q and Mono S chromatography. The Apaf-2 activity flowed through the Mono Q column. The flowthrough was loaded directly onto the Mono S column, and the bound Apaf-2 activity was eluted with a 100–300 mM NaCl linear salt gradient. The fractions from the Mono S column were collected and assayed. As shown in Figure 4A, the Apaf-2 activity eluted from the Mono S column at ~120 mM NaCl (fractions 2–4). These fractions were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by silver staining (Figure 4B). A protein of apparent molecular mass of 15 kDa was observed to co-elute with the activity. No other proteins were detected by silver staining in the active Apaf-2 fractions.

Table 1 shows quantitative estimates of the results of a complete purification of Apaf-2 starting with the S-100 fraction from 20 liters of HeLa cells, which contained 348.5 mg of protein. The protein was purified more than 2000-fold with an overall recovery of 152% activity. The >100% recovery indicates the elimination of inhibitory activities during the purification.

Identification of the 15-kDa Apaf-2 as Cytochrome c

Purified Apaf-2 had a noticeable pink color, which prompted us to measure its spectrophotometric absorbance (Figure 5). The protein showed absorbance peaks at 415, 520, and 549 nm, a spectrum shared by the reduced form of cytochrome c (Margoliash and Walasek, 1967). The identity was confirmed by amino acid sequences generated from tryptic peptides isolated from the 15-kDa Apaf-2. All the sequences show 100% identity with the reported sequence of human cytochrome c (Table 2).

To confirm that cytochrome c has Apaf-2 activity, we purchased cytochrome c purified from bovine heart and rat liver from a commercial source and tested them for Apaf-2 activity. As shown in Figure 6, cytochrome c from

Table 1. Purification of Apaf-2 from HeLa Cells

Step	Fraction	Protein	Specific Activity	Total Activity	Purification	Recovery
		mg	units/mg	unit	-fold	%
1	S-100	348.5				
2	Phosphocelluse	104	126.6	13166	1	100
3	50% Ammonium-Sulfate Precipitation	23.8	833.3	19824	6.6	150
4	Phenyl-Sepharose	0.473	42145	19934	333	151
5	Superdex-200	0.460	43367	19950	343	152
6	Mono Q/Mono-S	0.076	263150	20000	2079	152

S-100 was prepared from 20 liters of HeLa cells in spinner culture as described under Experimental Procedures. An aliquot of each fraction was dialyzed against buffer A and the Apaf-2 activity was assayed by recombining with ^{35}S-labeled CPP32 at four concentrations of protein. The results were quantified by PhosphorImaging.

both bovine heart and rat liver were able to initiate the dATP-dependent activation of CPP32 as efficiently as Apaf-2 (lanes 3–6).

Immunodepletion and Reconstitution of Cytochrome c–Dependent Apoptotic Program

To rule out the possibility that the Apaf-2 activity is due to a minor contaminating protein that copurified with cytochrome c, we performed an immunodepletion experiment using a monoclonal antibody against rat cytochrome c. This monoclonal antibody cross-reacts with purified Apaf-2 (data not shown). As shown in Figure 7, HeLa cell S-100 depleted of cytochrome c using the monoclonal anti-cytochrome c antibody lost the dATP-dependent activation of CPP32 and the ability to induce DNA fragmentation in the added nuclei (Figures 7A and 7C, lanes 3 and 4). Adding back either the purified Apaf-2 from HeLa cells or the commercial cytochrome c from bovine heart or rat liver to the immunodepleted extracts restored the dATP-dependent activation of CPP32 and DNA fragmentation (Figure 7A and 7C, lanes 5–10). The reconstitution of cytochrome c–dependent activation of CPP32 was evident with the addition of 0.01 μg (33 nM) of purified cytochrome c to the cytochrome c–depleted extracts (Figure 7B). Addition of 0.3 μg of cytochrome c recovered more than 100% of control activity, indicating that the cytochrome c in the cytosol is not at saturation level (Figure 7B). The dATP and cytochrome c–dependent activation of CPP32 and DNA fragmentation was accompanied by the morphological change in the co-incubated nuclei that is characteristic of apoptosis (Figure 7D).

To investigate whether the dATP and cytochrome c–dependent activation of CPP32 is a general phenomenon, we prepared cytosols from human embryonic kidney 293 cells and human monoblastic leukemia U937 cells. As shown in Figure 8, S-100 fractions from both cell types contained a dATP-dependent CPP32 activating activity (lanes 1, 2, 7, and 8). Immunodepletion of cytochrome c from these cytosols resulted in the loss of CPP32 activating activity (lanes 3, 4, 9, and 10), and addition of purified cytochrome c restored the activity (lanes 5, 6, 11, and 12).

Release of Cytochrome c to the Cytosol upon Apoptotic Stimulation

Human cytochrome c is encoded by a single copy nuclear gene (Evans and Scarpulla, 1988), which is trans-

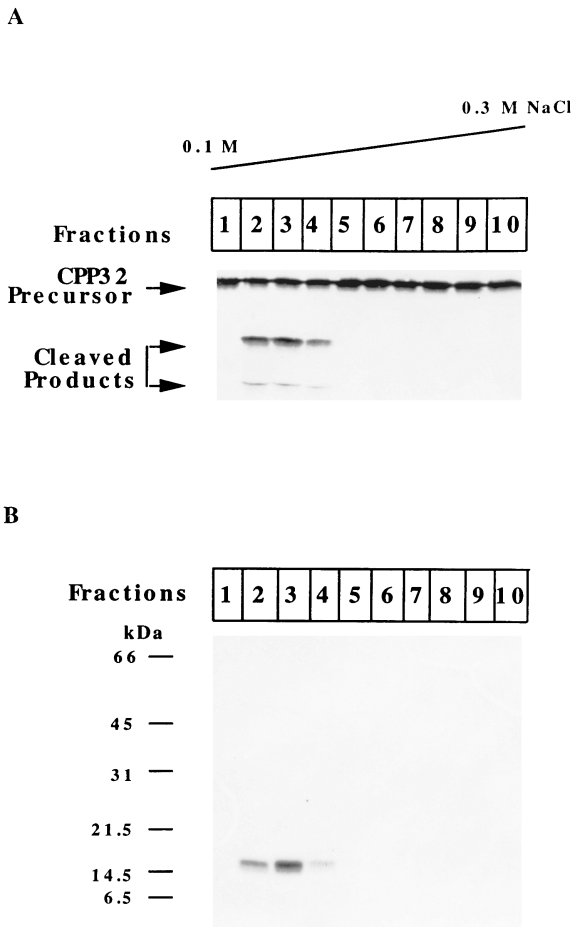

Figure 4. Mono S Column Purification of Apaf-2

The Apaf-2 activity that bound to the phosphocellulose column was purified through the Mono S column as described in the Experimental Procedures.

(A) Aliquots of 1 μl of Mono S column fractions were incubated with aliquots of 10 μl of phosphocellulose flowthrough fraction and 3 μl of in vitro translated, ^{35}S-labeled CPP32 at 30°C for 1 hr in the presence of 1 mM dATP in a final volume of 20 μl of buffer A. Samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter. The filter was exposed to film for 16 hr at room temperature.

(B) Aliquots (30 μl) of the Mono S fractions were subjected to 15% SDS–PAGE followed by silver staining.

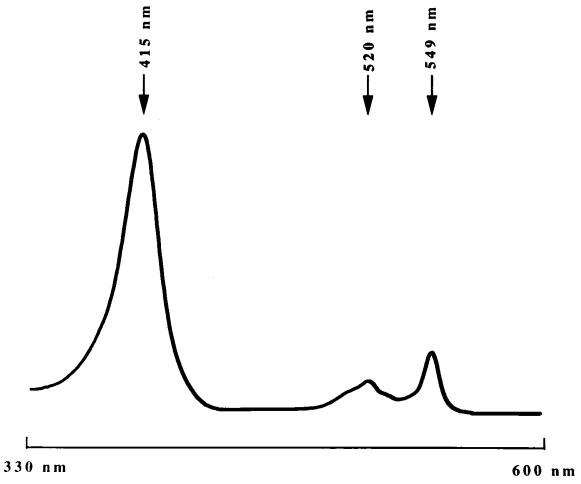

Figure 5. Absorption Spectrum of Apaf-2

Aliquot of 1 ml of Apaf-2 purified through the Mono S column was subjected to absorption spectrum scanning using a CARY 219 spectrophotometer. Absorption spectrum was recorded between 330 nm and 600 nm at a scanning speed of 1 nm/s.

lated on cytoplasmic ribosomes as apocytochrome c. The heme group of cytochrome c is attached to apocytochrome c upon its translocation into mitochondria (Gonzales and Neupert, 1990). The holocytochrome c is a soluble protein located in the intermembrane space of mitochondria (Gonzales and Neupert, 1990). The presence of cytochrome c in our cytosolic fraction therefore may be the result of ruptured outer mitochondrial membrane by hypotonic shock during its preparation. To test this hypothesis, we prepared cytosol from HeLa cells in the presence of 250 mM sucrose to protect the mitochondrial integrity. The cells were broken gently by douncing in a sandpaper-polished piston (Hayakawa et al., 1993). Cytosol prepared this way (designated S-cytosol) contained little cytochrome c compared with the cytosol used in the previous experiments (Figure 10A, lanes 1 and 2). As shown in Figure 9, S-cytosol was incapable of initiating the dATP-dependent activation of CPP32 (lanes 1 and 2) unless purified cytochrome c was added (lanes 3 and 4).

Table 2. Sequences of Tryptic Peptides from the 15-kDa Apaf-2: Comparison with Human Cytochrome c

Tryptic Peptide
1. EERADLIAY (89–96)
2. TGPNLHGLFGR (28–38)
3. TGQAPGYSYTAANK (40–53)
4. YIPGTK (74–79)
5. *II*GEDTLMEYL (56–68)
6. IFIMK (9–13)
7. TGPNL (28–32)

Sequences were obtained from Edman degradation performed on the HPLC-purified tryptic (Lys-C) peptides generated from the SDS-gel-purified 15-kDa Apaf-2. The sequence of human cytochrome c was reported by Evans and Scarpulla (1988). The asterisk denotes a residue in Apaf-2 that could not be assigned based on peptide sequence analysis. Numbers in parentheses denote the amino acid position in the cDNA sequence of human cytochrome c.

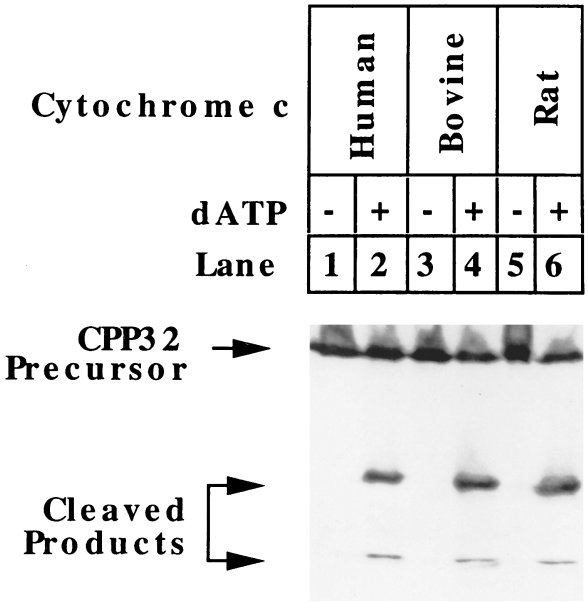

Figure 6. Cytochrome c from Bovine Heart and Rat Livers Have Apaf-2 Activity

Aliquots of 0.2 μg of Apaf-2 purified through the Mono S column (lanes 1 and 2), cytochrome c from bovine heart (lanes 3 and 4), and rat liver (lanes 5 and 6) were incubated with aliquots of 10 μl of phosphocellulose flowthrough fraction and 3 μl in vitro translated, ^{35}S-labeled CPP32 at 30°C for 1 hr in a final volume of 20 μl buffer A in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 1 mM dATP. Samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter, which was then exposed to a film for 16 hr at room temperature.

The requirement for cytochrome c in the apoptotic program in vitro suggests that there may be increased release of cytochrome c to the cytosol in cells undergoing apoptosis. To test this hypothesis, we treated HeLa cells with an apoptosis-inducing reagent, staurosporine. Staurosporine is a broad-spectrum inhibitor of protein kinases and has been found to be a potent apoptosis inducer in a variety of cell types (Rueggs and Burgess, 1989; Jacobson et al., 1993; Wang et al., 1996). Cytosol was prepared from staurosporine-treated cells using the sucrose-containing buffer, and the cells were dounced by the sandpaper-polished piston. As shown in Figure 10B, staurosporine treatment of HeLa cells resulted in activation of the endogenous CPP32 as detected by the cleavage of PARP. S-cytosol from staurosporine-treated HeLa cells contained a markedly elevated level of cytochrome c compared with that from nontreated cells (Figure 10A, lanes 2 and 3). The same phenomenon was also observed in human monoblastic U937 cells (data not shown).

Discussion

In Vitro System of Apoptosis

We report here the establishment of an in vitro system that faithfully duplicates the two best characterized biochemical markers of apoptosis, i.e., DNA fragmentation into nucleosomal fragments and the activation of the ICE-related apoptotic protease CPP32. This in vitro system allowed us to fractionate and begin to isolate the

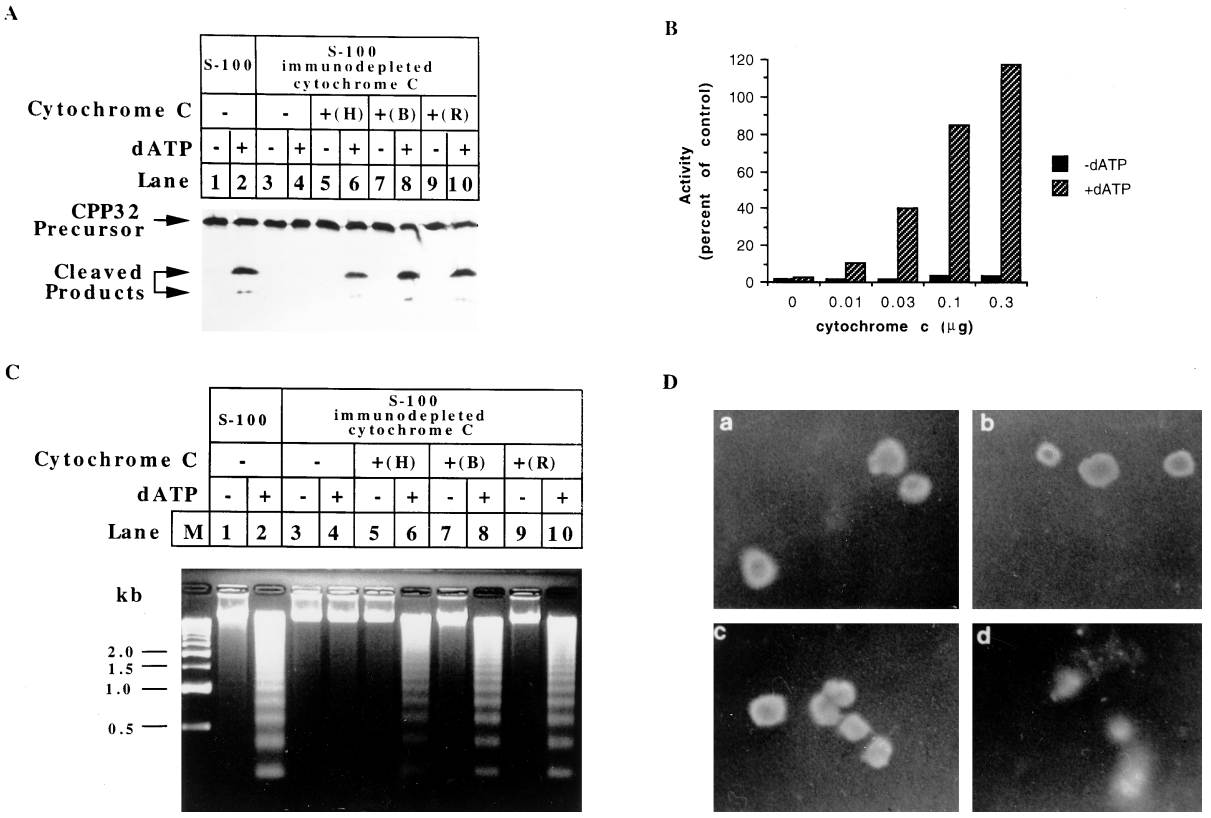

Figure 7. Immunodepletion of Cytochrome c from HeLa S-100 and Reconstitution of dATP-Dependent Activation of CPP32, DNA Fragmentation, and Nuclear Morphological Change Using Purified Cytochrome c

Cytochrome c present in the HeLa cell S-100 was immunodepleted as described in the Experimental Procedures.

(A) Aliquots of 10 μl of HeLa S-100 (50 μg) (lanes 1 and 2), or 10 μl of HeLa S-100 immunodepleted of cytochrome c (lanes 3 and 4), or 10 μl of HeLa S-100 immunodepleted of cytochrome c supplemented with 0.2 μg Apaf-2 purified through the Mono S column (H) (lanes 5 and 6), bovine heart cytochrome c (B) (lanes 7 and 8), or rat liver cytochrome c (R) (lanes 9 and 10), were incubated with aliquots of 3 μl of in vitro translated, ^{35}S-labeled CPP32 in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 1 mM dATP at 30°C for 1 hr in a final reaction volume of 20 μl of buffer A. Samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter, which was then exposed to film for 16 hr at room temperature.

(B) HeLa S-100 (50 μg) immunodepleted of cytochrome c were reconstituted with the indicated amount of Apaf-2 (purified through Mono S column step) in a CPP32 cleavage reaction as described in (A). The cleaved products (p20) were quantified in a Fuji-1000 PhosphorImager machine and plotted in comparison with that generated by HeLa S-100.

(C) Aliquots of 50 μl of HeLa S-100 (250 μg) (lanes 1 and 2), or 50 μl of HeLa S-100 immunodepleted of cytochrome c (lanes 3 and 4), or 50 μl of HeLa S-100 immunodepleted of cytochrome c supplemented with 1 μg of Apaf-2 purified through the Mono S column (H) (lanes 5 and 6), bovine heart cytochrome c (B) (lanes 7 and 8), or rat liver cytochrome c (R) (lanes 9 and 10), were incubated with aliquots of 6 μl of hamster liver nuclei in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 1 mM dATP at 37°C for 2 hr in a final reaction volume of 60 μl. The DNA was isolated as described in the Experimental Procedures and analyzed on 2% agarose gel electrophoresis. The DNA was visualized by ethidium bromide staining.

(D) DNA fragmentation assays were carried out as in (C) using HeLa S-100 immunodepleted of cytochrome c alone (a and b) or supplemented with Apaf-2 purified through Mono S column step (c and d) in the absence (a and c) or presence of 1 mM dATP (b and d). After a 2-hr incubation at 37°C, an aliquot of each reaction (30 μl) was stained with 4′,6′-diamidino-2-phenylindole (DAPI), and observed under a fluorescence microscope with a UV-2A combination filter.

required components. One required protein factor was purified to homogeneity and identified as the human cytochrome c.

This dATP- and cytochrome c–dependent in vitro apoptosis system seems to represent a general apoptotic program. Identical results were obtained from cytosols of HeLa cells, human embryonic kidney 293 cells, and human monoblastic U937 cells.

There have been several previous reports of cell-free apoptosis systems based on extracts from hormone-treated Xenopus eggs (Newmeyer et al., 1994), double synchronized mitotic chicken hepatoma cells (Lazebnik et al., 1993), or extracts from Fas, ultraviolet (UV)-irradiated, and ceramide-treated cells (Eeari et al., 1995; Martin et al., 1995). Our system differs from the previously reported sytems in that it uses extracts from normally growing cells that have not been induced to undergo apoptosis. This allows apoptosis to be initiated in vitro. Because it uses only soluble components, the system is amenable to fractionation and reconstitution. This has enabled us to identify factors that participate in the initiation of apoptosis.

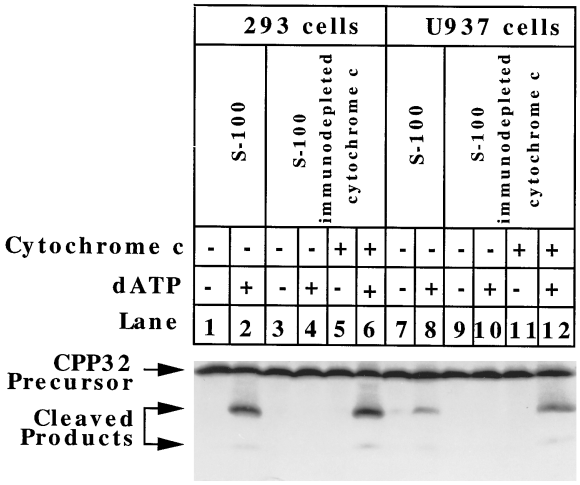

Figure 8. dATP and Cytochrome c–Dependent Activation of CPP32 in Cytosols from Human Embryonic Kidney 293 Cells and Human Monoblastic U937 Cells

S-100 fractions from 293 cells and U937 cells were prepared and their cytochrome c was immunodepleted as described in the Experimental Procedures. CPP32 activation reactions were carried out as described in Figure 7, except that 25 μg of S-100 was used in each reaction. We observed that 1 mM of dATP was present in lanes 2, 4, 6, 8, 10, and 12. Lanes 1 and 2, S-100 fraction from 293 cells; lanes 3 and 4, S-100 fraction from 293 cells immunodepleted of cytochrome c; lanes 5 and 6, S-100 fraction from 293 cells immunodepleted of cytochrome c supplemented with 0.2 μg of Apaf-2 purified through Mono S column step; lanes 7 and 8, S-100 fraction from U937 cells; lanes 8 and 9, S-100 fraction from U937 cells immunodepleted of cytochrome c; lanes 11 and 12, S-100 fraction from U937 cells immunodepleted of cytochrome c supplemented with 0.2 μg of Apaf-2 purified through Mono S column step.

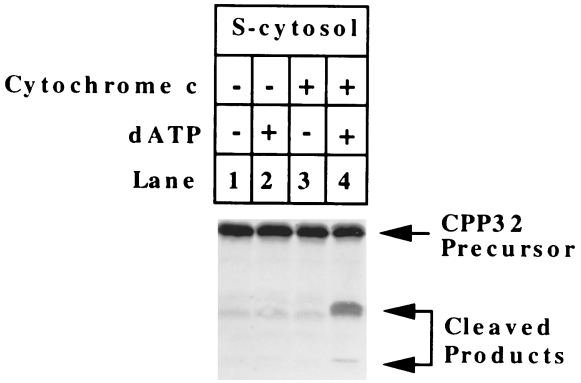

Figure 9. Reconstitution of dATP-Dependent Activation of CPP32 with S-cytosol and Purified Apaf-2

On day 0, Hela cells were set up at 5×10^5 cells per 100-mm dish in medium A as described in the Experimental Procedures. On day 2, cells were harvested, collected by centrifugation (1000 g for 10 min at 4°C). After being washed once with ice-cold PBS, the cell pellet was suspended in 5 vol of ice-cold buffer A containing 250 mM sucrose. The cells were disrupted by douncing 3 times in a 5-ml Wheaton douncer with the pestle polished by sandpaper. After centrifugation in a microcentrifuge for 5 min at 4°C, the supernatants were further centrifuged at $10^5 \times$ g for 30 min in a table top ultracentrifuge (Beckman). The resulting supernatants were designated as S-cytosol. Aliquots of S-cytosol (50 μg) alone (lanes 1 and 2), or supplemented with 0.2 μg of Apaf-2 purified through the Mono S column (lanes 3 and 4), were incubated with aliquots of 3 μl in vitro translated, ^{35}S-labeled CPP32 in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM dATP at 30°C for 1 hr in a final reaction volume of 20 μl of buffer A. Samples were subjected to SDS–PAGE and transferred to a nitrocellulose filter, which was then exposed to film for 16 hr at room temperature.

dATP/dADP as the Signal for Apoptosis

In vitro apoptosis in our system was initiated by the addition of dATP. Although our finding that dATP plays a critical role for initiation of apoptosis in vitro was empirical, dATP has long been implicated in cell death. The best known case is the inherited deficiency of adenosine deaminase (ADA), which results in severe combined immunodeficiency (SCID). In ADA patients, there is an abnormal accumulation of dATP up to mM level as measured in their erythrocytes and death of $CD8^{low}$ transitional and CD^4CD^8 double-positive thymocytes by an apoptosis mechanism (Cohen et al., 1978; Goday et al., 1985; Benveniste and Cohen, 1995). It also has been reported that deoxyadenosine treatment of cultured chick embryonic sympathetic neurons results in the accumulation of dATP and death through apoptosis (Wakade et al., 1995). Neuronal cell death was prevented by a nucleotide kinase inhibitor, suggesting that dATP accumulation was the cause of cell death (Wakade et al., 1995). Our finding that dATP can initiate the activation of CPP32 and DNA fragmentation provides a mechanistic explanation for the dATP-mediated cell toxicity. The finding that dADP can substitute for dATP is interesting. In cells treated with an ADA inhibitor, dADP also accumulates, although to a lesser extent than dATP (Goday et al., 1985). Whether dADP or dATP function by themselves, or whether they must be converted to the other, remains to be determined.

Cytochrome c as a Required Component of the Cell Suicide Program

The fractionation of the factors necessary for dATP-dependent activation of CPP32 resulted in the isolation of cytochrome c as one of the necessary components for apoptosis in vitro. It is unlikely that cytochrome c was mimicking the function of another protein, since cytochrome c is the only protein purified from the S-100 fraction that has Apaf-2 activity. The requirement for cytochrome c is confirmed further by the depletion and reconstitution experiments.

Cytochrome c is an essential component of the mitochondrial respiratory chain. It is a soluble protein that is localized in the intermembrane space and is loosely attached to the surface of the inner mitochondrial membrane (Gonzales and Neupert, 1990). Cytochrome c is translated in the cytoplasmic ribosomes and follows a unique pathway into mitochondria that does not require the signal sequence, electrochemical potential, and general protein translocation machinery (Mayer et al., 1995).

Mitochondria have been implicated in apoptosis ever since the discovery that the bcl-2 family of proteins is located in the outer mitochondrial membrane (Monaghan et al., 1992; Krajewski et al., 1993; de Jong et al., 1994). In vitro apoptosis in Xenopus egg extracts requires a dense organelle fraction enriched in mitochondria (Newmeyer et al., 1994). Purified mitochondria from hamster heart can supplement the cytosol immunodepleted of cytochrome c, or the cytosol prepared in

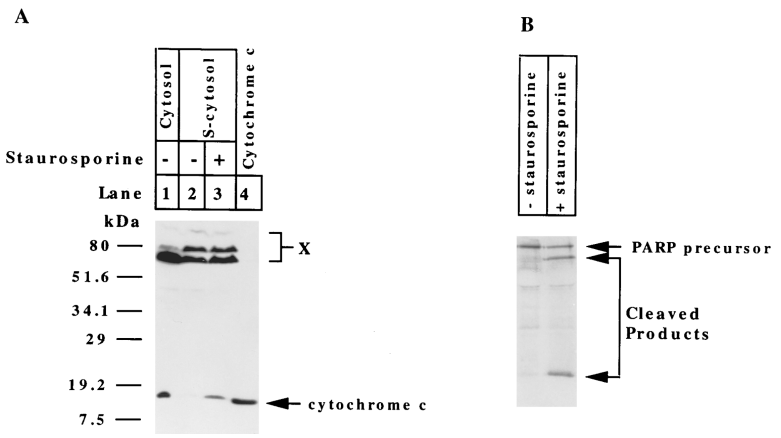

Figure 10. Increased Release of Cytochrome c to the Cytosol upon Apoptotic Stimulation

HeLa cells were set up as described in Figure 9. On day 2, staurosporine at a final concentration of 1 μM was added to the medium as indicated. After incubation at 37°C for 6 hr, the cells were harvested and S-cytosols were prepared as described in Figure 9.

(A) An aliquot (50 μg) of HeLa cell S-100 fraction used in Figures 1–7 (lane 1), or S-cytosol from HeLa cells (lane 2), or S-cytosol from HeLa cells treated with staurosporine for 6 hr. In lane 4, aliquot of 0.2 μg of Apaf-2 purified through Mono S column step. The samples were subjected to 15% SDS–PAGE and transferred to a nitrocellulose filter. The filter was probed with a monoclonal anti-cytochrome c antibody and the antigen/antibody complex was visualized by the ECL method as described in the Experimental Procedures. The filter was exposed to a Kodak X-OMAT AR X-ray film for 15 s. The arrow denotes the position of cytochrome c; the X denotes protein bands that cross-reacted with this antibody.

(B) Aliquots of 4.5 μg of S-cytosol from HeLa cells (−staurosporine) or HeLa cells treated with 1 μM staurosporine for 6 hr (+staurosporine) were incubated with aliquots of 10 μl of in vitro translated, ^{35}S-labeled PARP for 30 min at 30°C in a volume of 20 μl of buffer A. The samples were subjected to 12% SDS–PAGE and transferred to a nitrocellulose filter. The filter was exposed to film for 4 hr at room temperature.

the presence of sucrose, to support CPP32 activating reaction (J. Y. and X. W., unpublished data). However, one argument against the involvement of mitochondria in apoptosis comes from the results of Jacobson et al., who showed that apoptosis and bcl-2 protection of apoptosis is normal in cells that lack mitochondrial DNA (Jacobson et al., 1993). In addition, none of the known mitochondrial functions, such as ATP production, electron transfer, oxidative phosphorylation, generation of reactive oxygen species, and Ca^{2+} uptake, seem to account for its involvement in apoptosis (Jacobson et al., 1993; Hockenbery et al., 1993; Newmeyer et al., 1994). Our finding that cytochrome c is a necessary component of cellular apoptotic program suggests that mitochondria may be involved in apoptosis by releasing cytochrome c. Since cytochrome c is encoded by a nuclear gene and the translocation of apocytochrome c into mitochondria does not require membrane potential and general protein translocation machinery (Evans and Scarpulla, 1988; Mayer et al., 1995), it may be totally functional in apoptosis in cells lacking mitochondrial DNA. Consistent with this model, the cells undergoing apoptosis induced by staurosporine showed increased levels of cytochrome c in their cytosol. The release of cytochrome c into the cytosol could provide a potential target for regulation of apoptosis, possibly by the bcl-2 family of proteins.

The biochemical mechanism of cytochrome c function in the activation of CPP32 remains to be determined. Further understanding requires the purification and characterization of Apaf-1, which is another component required for the CPP32 activation reaction.

Experimental Procedures

General Methods and Materials

The nucleotide triphosphates were purchased from Phamacia; ADP, dADP, AMP, dAMP, adenosine, and deoxyadenosine were from ICN; pepstatin A, leupeptin, and N-acetyl-leucyl-leucyl-norleucina (ALLN) were from Boehringer Mannheim; phenylmethyl-sulphonyl fluoride (PMSF), imidazole, cAMP, aprotinin, bovine heart cytochrome c, and rat liver cytochrome c were from Sigma; ^{35}S-methionine was from Amersham Corporation; molecular weight standards for SDS–PAGE and gel-filtration chromatography were from Bio-Rad. cDNA clones of human SREBP-2 and hamster CPP32 were described in the indicated references (see below). Protein concentration was determined by the Bradford Method. Silver staining was carried out using a Silver Stain Plus kit from Bio-Rad. Plasmids were purified using a Megaprep kit from Qiagen.

Preparation of S-100 Fractions from HeLa cells, 293 Cells, and U937 Cells

Human HeLa S3 cells were grown as described (Wang et al., 1993). The cells (5×10^5/ml) were harvested by centrifugation at 1800 × g for 10 min at 4°C. After being washed once with ice-cold phosphate buffered saline (PBS), the cell pellet was suspended in 5 vol of ice-cold buffer A (20 mM Hepes–KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol [DTT], and 0.1 mM PMSF), supplemented with protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml ALLN). After sitting on ice for 15 min, the cells were disrupted by douncing 15 times in a 100 ml Kontes douncer with the B pestle (Kontes Glass Company). The nuclei were centrifuged at 1000 × g for 10 min at 4°C. The supernatant was further centrifuged at 10^5 × g for 1 hr in a Beckman SW 28 rotor. The resulting supernatant (S-100 fraction) was stored at −80°C and used for the in vitro apoptosis assay and the starting material for the purification of Apaf-2.

We set up 293 cells at 5×10^5 cells per 100 mm dish in medium A (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% [v/v] heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate). After incubation for 48 hr at 37°C in a 5% CO_2 incubator, the cells were harvested, collected by centrifugation (1000 g for 10 min at 4°C). U937 cells were set up at 5×10^5 cells/ml in medium B (RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate). After incubation for 48 hr in a 5% CO_2 incubator, the cells were collected by centrifugation (1000 g for 10 min at 4°C). The cell pellets of 239 cells and U937 cells were washed once with ice-cold PBS and resuspended in 5 vol of ice-cold buffer A supplemented with protease inhibitors. After sitting on ice for 15 min, the cells were broken by passing 15 times through a G22 needle. After centrifugation in a microcentrifuge for 5 min at 4°C, the supernatants were further centrifuged at 10^5 × g for 30 min in a table top ultracentrifuge (Beckman). The resulting supernatants were used for the in vitro apoptosis assay.

In Vitro Translation of CPP32, SREBP, and PARP

A polymerase chain reaction fragment encoding amino acids 29–277 of hamster CPP32 (Wang et al., 1996) was cloned into NdeI and

BamHI sites of pET 15b vector (Novagen). The resulting fusion protein of six histidines with hamster CPP32 (amino acids 29–277) was translated in a TNT T7 transcription/translation kit (Promega) in the presence of ^{35}S-methionine according to the instructions of the manufacturer. The translated protein was passed through a 1-ml nickel affinity column (Qiagen) equilibrated with buffer A. After washing the column with 10 ml of buffer A, the translated CPP32 was eluted with buffer A containing 250 mM imidazole. Human SREBP-2 was translated in a TNT SP6 transcription/translation kit as described (Wang et al., 1995). Full-length human PARP cDNA was cloned into SmaI and EcoRI sites of pBK-CMV vector (Stratagene) and translated in a TNT T7 transcription/translation kit. The translated SREBP-2 and PARP (200 μl each) were purified by passing each translation mixture through a 10-ml Sephadex G-25 gel-filtration column equilibrated with buffer A. The translated proteins contained within the exclusion volume of the column were collected.

Western Blot Analysis

A monoclonal antibody against human CPP32 was purchased from Transduction Laboratories, and a monoclonal antibody against cytochrome c (7H8.2C12) was obtained as described previously (Jemmerson and Johnson, 1991). Immunoblot analysis was performed with horseradish peroxidase–conjugated anti-mouse immunoglobulin G using the Enhanced Chemiluminescence (ECL) Western Blotting Detection reagents (Amersham).

Assay for dATP-Dependent Activation of CPP32 Protease

CPP32 was translated and purified as described above. Aliquot of 3 μl of the in vitro translated CPP32 was incubated with the indicated protein fraction, nucleotides, and 1 mM additional $MgCl_2$ at 30°C for 1 hr in a final volume of 20 μl of buffer A. At the end of the incubation, 7 μl of 4× SDS sample buffer was added to each reaction. After boiling for 3 min, each sample was subjected to a 15% SDS–PAGE. The gel was transferred to a nitrocellulose filter that subsequently was exposed to a Kodak X-OMAT AR X-ray film for 16 hr at room temperature.

Purification of Apaf-2 from HeLa S-100

All purification steps were carried out at 4°C. All the chromatography steps except the phosphocellulose column were carried out using an automatic fast protein liquid chromatography (FPLC) station (Pharmacia).

We applied 85 ml of HeLa S-100 to a phosphocellulose column (40 ml bed volume) equilibrated with buffer A. The column was washed with three column volumes of buffer A and eluted with two column volumes of buffer A containing 0.5 M NaCl. Ammonium sulfate (50%) was added directly to the phosphocellulose 0.5 M eluate. After rotating at 4°C for 1 h, the mixture was centrifuged at 15,000 rpm for 15 min in a JA 20 rotor (Beckman). The supernatant was directly applied to a 10 ml phenyl sepharose column equilibrated with buffer A containing 50% ammonium sulfate. The column was washed with two bed volumes of buffer A containing 50% ammonium sulfate and eluted with buffer A containing 1 M ammonium sulfate. The eluate was loaded onto a Superdex-200 gel filtration column (300 ml) equilibrated with buffer A and eluted with the same buffer. Fractions of 10 ml were collected and assayed for Apaf-2 activity. The active fractions from the gel-filtration column were pooled and loaded onto a Mono Q 5/5 and a Mono S 5/5 column connected together. The columns were pre-equilibrated with buffer A. After loading, the columns were disconnected, and the Mono S column was washed with 5 ml of buffer A containing 0.1 M NaCl and the Apaf-2 activity was eluted from the column with a 20-ml 0.1 M–0.3 M linear NaCl gradient. Fractions of 1 ml were collected.

Preparation of Hamster Liver Nuclei

Livers from four male Golden Syrian hamsters (Sasco) were rinsed with ice-cold PBS and homogenized in 0.25 g/ml buffer B (10 mM Hepes–KOH [pH 7.6], 2.4 M sucrose, 15 mM KCl, 2 mM sodium EDTA, 0.15 mM spermine, 0.15 mM spermidine, 0.5 mM DTT, and 0.5 mM PMSF) by three strokes of a motor-driven homogenizer. The homogenates were centrifuged through a 10-ml cushion of buffer B at 25,000 rpm for 1 hr in a SW 28 rotor at 4°C. The nuclei pellet was resuspended in buffer C (10 mM PIPES [pH 7.4], 80 mM KCl, 20 mM NaCl, 5 mM sodium EGTA, 250 mM sucrose, and 1 mM DTT) at 8.5×10^7 nuclei/ml and stored at −80°C in multiple aliquots.

DNA Fragmentation Assay

Aliquots of 50 μl of HeLa cell S-100 and 6 μl of hamster liver nuclei were incubated at 37°C for 2 hr with 1 mM additional $MgCl_2$ in the absence or presence of 1 mM indicated nucleotide. After incubation, an aliquot of 500 μl of buffer D (100 mM Tris–HCl [pH 8.5], 5 mM EDTA, 0.2 M NaCl, 0.2% w/v SDS, and 0.2 mg/ml proteinase K) was added to each reaction and incubated at 37°C overnight. NaCl was then added to a final concentration of 1.5 M and the nuclear debris was spun down for 15 min in a microcentrifuge at room temperature. The DNA in the supernatant was precipitated with an equal volume of 100% (v/v) ethanol. The DNA precipitate was washed once with 70% ethanol and resuspended in 40 μl of buffer E containing 10 mM Tris–HCl, (pH 7.5), 1 mM sodium EDTA, and 200 μg/ml DNase-free RNase A (Worthington). After incubation at 37°C for 2 hr, the DNA was loaded onto a 2% agarose gel and electrophoresis was conducted at 50 V for 2 hr in 0.5 × Tris–borate–EDTA (TBE) buffer (1 × TBE buffer contains 90 mM Tris–borate/2 mM EDTA). The gel was stained with 2 μg/ml ethidium bromide for 15 min, destained with water for 1 hr, and visualized under UV light.

Immunodepletion of Cytochrome c from HeLa S-100

An anti-cytochrome c antibody (6H2. B4) that recognizes the native form of cytochrome c was described previously (Jemmerson et al., 1991). An aliquot of 100 μl (0.7 mg/ml IgG 2A) of this antibody was incubated with a 1:1 mixture of 50 μl protein A and protein G agarose beads resuspended in 200 μl of PBS (Santa Cruz) at 4°C for 3 hr. The beads were collected by centrifugation for 15 min in a microcentrifuge at 4°C. After removal of the supernatant, the beads were washed once with 1 ml of buffer A and incubated with 1.5 ml S-100 fractions for 5 hr in a rotator at 4°C. The beads were subsequently pelleted by centrifugation for 15 min in a microcentrifuge at 4°C. The supernatant was used as S-100 immunodepleted of cytochrome c.

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